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REMARKS

The Office Action dated January 8, 2004 has been carefully reviewed. Applicants thank the Examiner for indicating that claims 33-35, 42 and 60-65 are allowed. In view of the foregoing amendments and the following remarks, Applicants respectfully request reconsideration and reexamination of this application and the timely allowance of the pending claims.

Claims 37-38 have been newly amended to correct minor informalities, as requested by the Examiner.

No new matter has been added by these claims and entry is respectfully requested. As a result of these amendments, claims 33-40, 42 and 60-65 are presently before the Examiner.

Response to the objections to the claims due to informalities

The Examiner has maintained the rejection of claim 37 because "it starts with an improper article" Applicants have corrected the informality in claim 37. Therefore, the Examiner is respectfully requested to withdraw the objections.

Response to the rejections under 35 U.S.C. § 112 (first paragraph)

Claims 36-40 stand rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the enablement requirement. Applicants respectfully traverse the rejection.

According to the rejection, *E. coli* strain KV832 is essential to the claimed invention and must be obtainable by a repeatable method set forth in the specification or otherwise be readily available from the public. The Office, in asserting that a deposit is required for enablement purposes, alleges the specification fails to disclose a repeatable process to obtain the KV832 strain and argues it is not apparent if the strain is readily available to the public.

In response to Applicants' detailed and considered explanation of why the rejection should not be maintained, the Office Action alleges that "Kiel et al could not be considered because it was not sent" and that "Kiel *et al* is cited on pg 13 of the substitute specification, not page 14."

First, Applicants direct the Examiner's attention to the filing postcard stamped by the U.S. Patent & Trademark Office ("U.S. PTO") Customer Window showing that the Amendment and Response was filed on September 29, 2003. This stamped post card clearly indicates that as-filed item number 6 was "Exhibit A – Copy of the article by Kiel *et al.*". Furthermore, page 2 of the accompanying Transmittal Form clearly shows under paragraph number 4 that the Kiel *et al.* article was filed with the Amendment and Response. In addition, page 13 of the Amendment and Response states the following: "Attachment: Exhibit A (Kiel *et al.*, 1987)". Thus, Applicants noted the attachment of the Kiel *et al.* article at least **three times** in the papers filed on September 29, 2003. Also, the copies of the as-filed papers sent to the Applicants by Applicants' Representative included a copy of the Kiel et al. article, as do the copies of the as-filed papers contained in Applicants' Representative's file. It is Applicants' Representative's normal course of business method to make an exact copy of the papers actually filed with the US PTO at the time the papers are finalized for filing. Therefore, all evidence clearly indicates that the Kiel *et al.* article was actually filed at the US PTO on September 29, 2003.

Applicants are disappointed that the Examiner was unable or unwilling to call Examiner's Representative and request a courtesy copy of the Kiel *et al.* article, which Applicants' Representative would have been more than willing to send to the Examiner immediately. Surely, this is not too great a burden on the Examiner since it is the US PTO that appears to have lost Applicants' as-filed papers. As it is, Applicants have now been unnecessarily harmed by the US PTO's loss of the Kiel *et al.* article and the Examiner's inability/unwillingness to request a copy of the as-filed article. As a result, Applicants will suffer a loss of time and money in responding to the "Final" Office Action for which the major outstanding issue deals with the teachings of the Kiel *et al.* paper lost by the Office.

Attached please find another copy of the as-filed Kiel *et al.* article. The Examiner is respectfully requested to call Applicants' representative if this second copy of the article did not arrive at her desk so that she could consider this timely-filed response.

As regards the referenced page number, Applicants have noted that the Examiner found the reference on page 13 instead of page 14.

Contrary to the arguments of the maintained rejection from the previous Office Action, the specification does in fact disclose a repeatable process to obtain the KV832 strain. Applicants

bring the document by Kiel *et al.* (Kiel *et al.*, "A general method for the construction of *Escherichia coli* mutants by homologous recombination and plasmid segregation," Mol. Gen. Genet. 207: 294-301 (1987)) [Kiel] to the attention of the Office (second copy attached as Exhibit A). See, specification page 13 citing Kiel *et al.*

According to Kiel, prior to the publication of the article, production of *E. coli* mutants was limited due to the need for special *E. coli* strains, special plasmids and special phages. Kiel's publication is an advancement in the art since the publication teaches a general method for obtaining mutants of *E. coli* that does not involve the use of such special strains, plasmids or phages, thus simplifying the introduction of mutations into the *E. coli* chromosome. The method makes use of the fact that many recombinant multicopy plasmids are segregationally unstable in *E. coli*, thus allowing mutants to be easily isolated by selection for cells in which the mutant gene (marked by an antibiotic resistance marker) has been integrated into the chromosome and from which the donating plasmid has been lost.

The Kiel method involves construction of plasmid pKVB2, a pBR322 based plasmid, which carries the complete *glg*B gene. The plasmid is then transformed into *E. coli* JM83, an *E. coli* strain which is publicly available. Plasmid pBR322 is well suited for use in the method since a) the plasmid is a well known, commonly used plasmid in which many genes have already been cloned ; b) it contains a Tcr gene which allows plasmid-free cells to be easily detected; and, c) recombinant plasmids based on pBR322 are segregationally unstable in *E. coli*.

The Office Action newly states that "the specification does not teach the mutation in glgB that is present in KV832". It is not necessary that the specification teach that this mutation is present. The specification teaches that the KV832 strain was used for the complementation analyses and the reference article teaches a repeatable method to produce KV832. The "name" or "designation" of the specific mutation is irrelevant to the invention as disclosed and claimed.

Thus, the Kiel publication describes a general method which allows mutants to be simply and efficiently isolated via gene replacement and plasmid loss. It is clear from Kiel *et al.* that one of ordinary skill in the art would be able to produce *E. coli* KV832 by a readily repeatable process known in the art. Therefore, since the specification is enabling for the production of *E. coli* KV832, no deposit is required. In view of the arguments presented above, reconsideration and withdrawal of the rejection is respectfully requested.

Response to the rejection under 35 U.S.C. § 112, second paragraph

Claim 38 remains rejected under 35 U.S.C. § 112, second paragraph, as purportedly being indefinite for failing to particularly point out and distinctly claim the subject matter which the Applicants regard as the invention. Applicants have corrected the alleged indefiniteness in claim 38. Therefore, the Examiner is respectfully requested to withdraw the requirement.

Conclusion

The foregoing amendments and remarks are being made to place the application in condition for allowance. Applicants respectfully request reconsideration and the timely allowance of the pending claims. A favorable action is awaited. Should the Examiner find that an interview would be helpful to further prosecution of this application, she is invited to telephone the undersigned at his convenience.

If there are any additional fees due in connection with the filing of this response, please charge the fees to our Deposit Account No. 50-0310. If a fee is required for an extension of time under 37 C.F.R. § 1.136 not accounted for above, such an extension is requested and the fee should also be charged to our Deposit Account.

Respectfully submitted,

MORGAN, LEWIS & BOCKIUS LLP

Date: March 12, 2004 By: 

Erich E. Veitenheimer
Reg. No. 40,420

Attachment: Second copy of Kiel *et al.*, 1987

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Mol Gen Genet (1987) 207:294–301

MGG

A general method for the construction of *Escherichia coli* mutants by homologous recombination and plasmid segregation

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Summary. A technique is presented by which mutations can be introduced into the *Escherichia coli* chromosome by gene replacement between the chromosome and a plasmid carrying the mutant gene. The segregational instability of plasmids in *E. coli* is used with high efficiency to isolate *E. coli* mutants. The method should be applicable to construction of mutants for any *E. coli* chromosomal gene provided it is dispensable, and for any *E. coli* strain provided it is capable of homologous recombination. The use of the method was demonstrated by constructing *E. coli* mutants for the glycogen branching enzyme gene (*glgB*) and the β-galactosidase gene (*lacZ*). The results show that recombination occurs via a reciprocal mechanism indicating that the method should, in a slightly modified form, also be useful in transferring chromosomal mutations onto multicopy plasmids in vivo.

Key words: *Escherichia coli* – Fusaric acid selection – Gene replacement – Plasmid segregation – Reciprocal recombination

Introduction

Using recombinant DNA techniques it has become possible to create mutations in vitro, which subsequently may be introduced into the *Escherichia coli* chromosome. Until now there have been limitations in the production of *E. coli* mutants by in vivo recombination techniques because either special *E. coli* strains (*polA*, e.g. Gutterson and Koshland 1983; *polA*^{ts}, e.g. Gay 1984; *recBCsbcB*, e.g. Jason and Schimmel 1984), special plasmids (temperature-sensitive origin of replication, Matsuyama and Mizushima 1985) or special phages (thermoinducible λ phage lacking the normal *att* site, Joyce and Grindley 1984) were required to introduce the mutation into the chromosome. A general method that does not involve the use of such special strains, plasmids or phages would simplify the introduction of mutations into the *E. coli* chromosome. This paper describes the development of such a system based on the segregational instability of recombinant, multicopy plasmids in *E. coli*.

Plasmid segregation has been demonstrated for a number of multicopy plasmids, e.g. pBR322 and its derivatives (Skogman et al. 1983; Jones et al. 1980) and pACYC184 and its derivatives (Summers and Sherratt 1984). It has been suggested (Summers and Sherratt 1984) that partitioning of multicopy plasmids is random and that plasmid-free cells are formed during the division of cells with a low copy number. Others have proposed the existence of an active partitioning function (Hashimoto-Gotoh and Ishii 1982; Della Latta et al. 1978).

It has been demonstrated that multimerization of multicopy plasmids (Summers and Sherratt 1984) and the insertion of large chromosomal DNA fragments (Jones et al. 1980) resulted in segregationally more unstable plasmids due to a decrease in copy number. This paper reports the use of these observations for the construction of *E. coli* mutants by a two-step process: (i) homologous recombination between the chromosome and the plasmid carrying a mutant gene inactivated by the insertion of an antibiotic-resistance marker, (ii) plasmid segregation allowing the simple and efficient isolation of mutants via a selection for the antibiotic-resistance marker in the (now chromosomal) mutant gene.

This method was tested by the construction of *E. coli* mutants carrying a deletion in the glycogen branching enzyme gene (*glgB*) using a phenotypic selection. The glycogen branching enzyme introduces α, 1–6 branches in linear α, 1–4 polyglucose chains during the synthesis of glycogen, a storage polymer (for review see Preiss 1984). The method was optimized to make it available for the construction of mutants for any non-essential gene in *E. coli* and the use of this optimized method was demonstrated by the construction of mutants for the β-galactosidase gene (*lacZ*).

Materials and methods

Bacteria and plasmids. *E. coli* K-12 strains and plasmids used and constructed in this study are listed in Table 1. Only the antibiotic-resistance markers of the plasmids are mentioned. Other genes present on these plasmids are shown in Figs. 2 and 3 and are described in the text.

Media. TY broth contained (per liter): 10 g tryptone, 5 g yeast extract, 10 g NaCl, pH 7.4. When necessary 0.5% glucose, 1.5% agar or 40 μg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside (Xgal) were added. Antibiotics were added as required: Tc 10 μg/ml, Ap 50 μg/ml, Km 50 μg/ml. Fusaric acid selection plates were used with slight modifica-

Offprint requests to: J.A.K.W. Kiel

Table 1. Bacteria and plasmids

Strain	Phenotype or genotype	Source or reference
Escherichia coli K-12		
BHB2600	803 *supE supF* $r^- m^+$ *met*	K. Murray
JM83	F^- *ara Δlac-pro thi strA* φ80d*lacZ*ΔM15	Vieira and Messing (1982)
KV832	JM83 with deletion *ΔglgB*1200 in the *glgB* region	This study
GM48	F^- *thr leu thi lacY galK galT mtl tonA tsx dam3 dcm6 supE*	Marinus (1973)
MC1000	F^- *ara* D139*Δ*(*araABC-leu*)7679 *galU galK ΔlacX*74 *rpsL thi*	Weinstock et al. (1983)
C600	F^- *thr leu thi lacY tonA supE*	Laboratory collection
KV6001	C600 *lacZ* by insertion of Km^r gene	This study
KV6002	C600 *lacZ* by insertion of Km^r gene	This study
Plasmids		
pJH1	Str^r Em^r Tc^r Km^r	Trieu-Cuot and Courvalin (1983)
pUC7	Ap^r	Vieira and Messing (1982)
pKM1	Ap^r Km^r	This study
pBR322	Ap^r Tc^r	Laboratory collection
pAD1	Ap^r Tc^r	Haziza et al. (1982)
pKVB1	Ap^r	This study
pOP190	Tc^r	Okita et al. (1981)
pKVB2	Tc^r Km^r	This study
pMLB1034	Ap^r	Casadaban and Cohen (1980)
pKVZ1	Ap^r Km^r	This study
pKVZ2	Ap^r Km^r	This study
pKVZ11	Ap^r Tc^r Km^r	This study
pKVZ12	Ap^r Tc^r Km^r	This study
pKVZ21	Ap^r Tc^r Km^r	This study
pKVZ22	Ap^r Tc^r Km^r	This study

tions as described by Maloy and Nunn (1981) and contained (per liter): 15 g agar, 5 g tryptone, 5 g yeast extract, 10 g NaCl, 10 g $NaH_2PO_4 \cdot H_2O$, 6 mg fusaric acid and 14 mg $ZnCl_2$.

Chemicals. Biochemicals (restriction enzymes, T_4 ligase and DNA polymerase I) were obtained from Boehringer, Mannheim (FRG) and were used according to the instructions of the manufacturer. All other chemicals were of analytical grade.

DNA preparations. Chromosomal DNA was extracted from *E. coli* as follows: cell pellets from 50 ml overnight cultures were suspended in 3 ml 25 mM Tris-Cl pH 7.8; 10 mM EDTA; 50 mM glucose; 1 mg/ml lysozyme. After incubation for 15 min at 0° C the cells were lysed by the addition of sarkosyl (1%). After 15 min incubation at 37° C, RNAse I (100 µg/ml; BDH, Poole, England) was added and incubation at 37° C was continued for another 30 min. Then preincubated pronase (1 mg/ml; Calbiochem, La Jolla, Ca.) was added. The mixture was incubated at 37° C until the lysate had cleared (approximately 1 h). Phenol extraction and ethanol precipitation were used to purify the DNA. Finally the DNA was dissolved in 10 mM Tris-Cl pH 7.8; 1 mM EDTA at a concentration of about 100 µg/ml. Preparative amounts of plasmid DNA were obtained according to the alkaline lysis procedure described by Maniatis et al. (1982). The analytical '*miniprep*' procedure described by Ish-Horowicz and Burke (1981) was used to extract plasmids from 2 ml cultures of *E. coli*.

Molecular cloning procedures. Routine DNA manipulations (restriction, ligation, transformation, agarose gel electrophoresis) were performed as described by Maniatis et al. (1982). DNA restriction fragments were isolated from agarose gels with hydroxylapatite (Bio-rad, Richmond, Ca.) according to Tabak and Flavell (1978).

Southern blot analysis. Southern transfer was carried out by electro-blotting DNA restriction fragments on Genescreen plus filters (Dupont NEN, Boston, Ma.) according to the instructions of the manufacturer. Filters were hybridized overnight at 65° C with α-^{32}P-dCTP (3000 Ci/mmol, Radiochemical Centre, Amersham) labelled probes (about 10^8 cpm/µg) in 5 × SSC; 1% SDS; 0.02% polyvinylpyrrolidone; 0.02% bovine serum albumin; 0.02% ficoll 400 and 250 µg/ml denatured calf thymus DNA. After hybridization, filters were washed twice at room temperature with 2 × SSC for 5 min, twice at 65° C with 2 × SSC; 0.5% SDS for 30 min and twice at room temperature with 0.1 × SSC for 30 min. The filters were then air-dried and autoradiographed.

Assay of segregational plasmid instability. Aliquots of 5 ml TY medium containing the appropriate antibiotics were inoculated with single colonies. Cultures were grown for approximately 5 generations at 37° C with vigorous shaking. The cultures were subsequently subcultured for approximately 100 generations in antibiotic-free medium. All cultures were diluted 1000-fold into 50 ml TY medium every 10 generations. Samples taken at appropriate time intervals were plated on selective TY plates (Tc) to determine the number of plasmid containing cells, and on TY plates to determine the total number of viable cells.

In vivo detection of glycogen branching enzyme activity. To initiate glycogen production *E. coli* cells were plated on TY plates supplemented with 0.5% glucose. After incubation for 24 h at 37° C, the colonies were flooded with iodine solution (0.01 M I_2, 0.03 M KI; 5 ml per plate) and stained

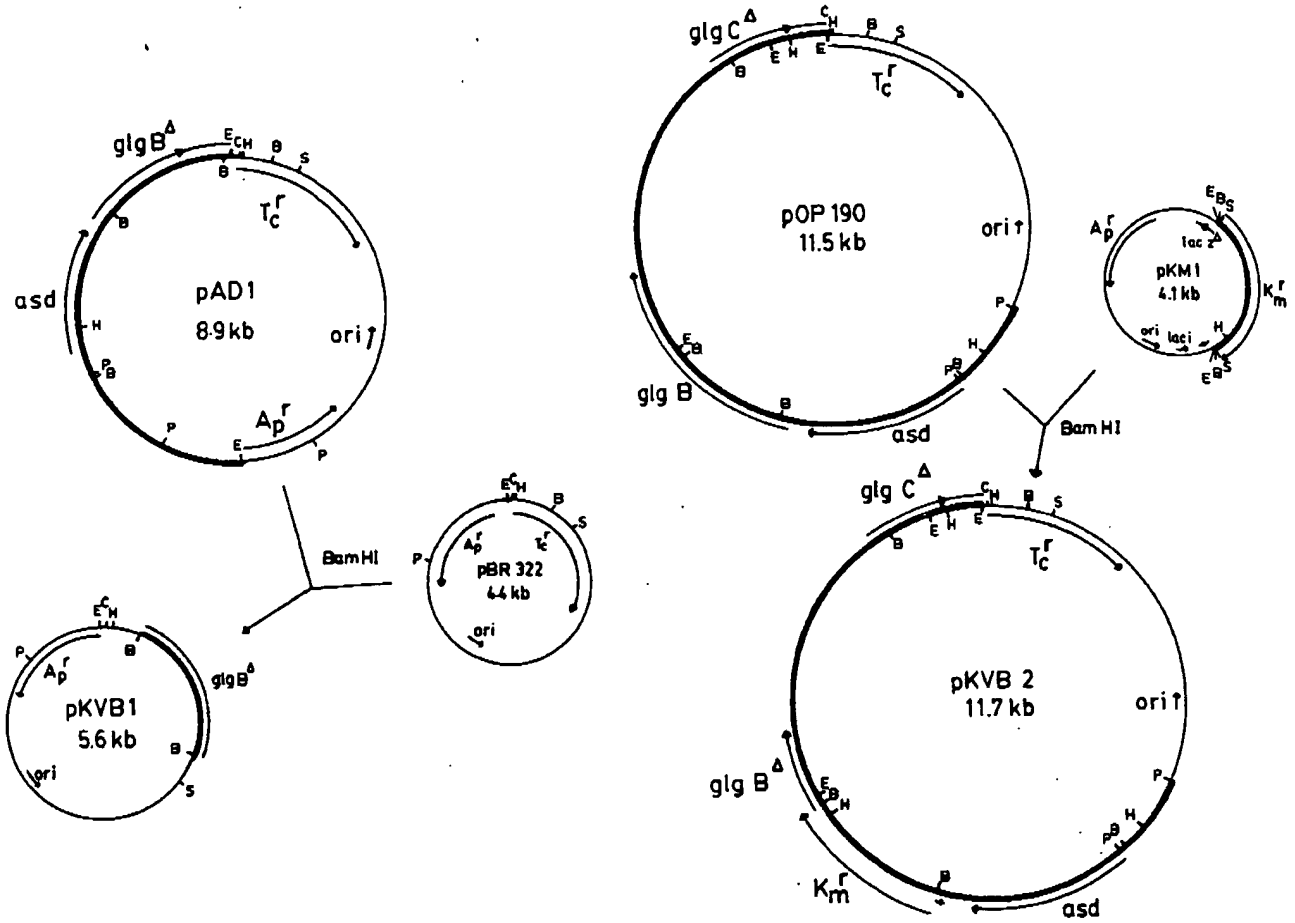

Fig. 1. Construction of plasmids pKVB1 and pKVB2. Plasmids pKVB1 and pKVB2 were constructed as described in Materials and methods. *glgBΔ*, *glgCΔ* and *lacZΔ* indicate the presence of a truncated gene. *asd*, aspartate semialdehyde dehydrogenase; *glgB*, glycogen branching enzyme; *glgC*, ADP-glucose pyrophosphorylase; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I

for approximately 30 s as described by Govons et al. (1969). Brown staining colonies had wild-type branching enzyme activity. Blue staining colonies were identified as glycogen branching enzyme deficient mutants (Damotte et al. 1968).

Detection of β-galactosidase activity. Cells were spread on TY plates containing Xgal (40 µg/ml). β-Galactosidase producing colonies were blue and β-galactosidase deficient mutants were white after overnight incubation at 37° C (Silhavy et al. 1984).

Fusaric acid selection. Tetracycline-sensitive *E. coli* cells were selected directly with a modification of the fusaric acid (FA) technique described by Bochner et al. (1980) and Maloy and Nunn (1981). To reduce carry-over of nutrients from the culture to the selective plates, the cultures were diluted 100-fold in minimal M9 medium (Miller 1972) before plating on the FA selection plates. After incubation for 48 h at 37° C the FA^r colonies were tested for their Tc-sensitivity by tooth-picking onto TY plates containing Tc.

Construction of plasmids pKVB1 and pKVB2 (Fig. 1). Plasmid pKM1 was constructed by cloning a filled in 1.4 kb *Hae*II fragment containing the Km^r gene from the *Streptococcus faecalis* plasmid pJH1 in the filled in *Pst*I site of pUC7. *E. coli* BHB2600 transformants containing pKM1 were selected on Ap- and Km-containing agar plates. The construction of this plasmid allows the simple isolation of the Km^r gene as a *Bam*HI, *Eco*RI, *Hin*cII, *Sal*I or *Acc*I fragment owing to the symmetrical multiple cloning site in pUC7.

Plasmid pKVB1 was constructed by ligation of the 1.2 kb *Bam*HI fragment from plasmid pAD1 that contains part of the *E. coli* glycogen branching enzyme gene (*glgB*) into the *Bam*HI site of pBR322. *E. coli* BHB2600 transformants containing pKVB1 were selected on Ap-containing agar plates.

For the construction of plasmid pKVB2, plasmid pOP190, an *Eco*RI deletion derivative of pOP12 (Okita et al. 1981) was used. pOP190 contains an insert of approximately 8 kb of *E. coli* chromosomal DNA which carries the complete *glgB* gene. The plasmid was partially digested with *Bam*HI. After agarose gel electrophoresis, fragments of approximately 10 kb were isolated and ligated to the 1.4 kb *Bam*HI fragment bearing the Km^r gene from pKM1. *E. coli* JM83 transformants carrying pKVB2 were selected on Tc- and Km-containing plates. In plasmid pKVB2 the 1.2 kb *Bam*HI fragment from the *E. coli glgB* gene (the same fragment that had been subcloned in plasmid pKVB1) was replaced by the Km^r marker. This Km^r gene is orientated in a clockwise fashion in the *E. coli glgB* gene.

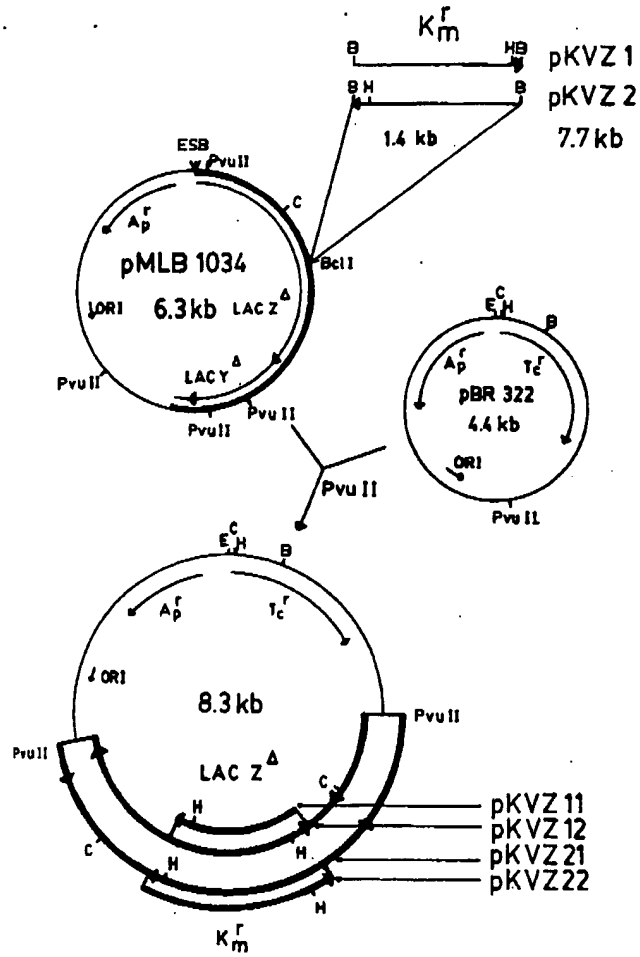

Fig. 2. Construction of the pKVZ plasmids. The construction of these plasmids is described in detail in Materials and methods. *Thin lines* indicate vector DNA (pBR322), *thick lines* insert DNA (*lacZ* and Km^r genes). LacZΔ, and LacYΔ indicate the presence of a truncated gene. B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; S, *Sma*I

Construction of the pKVZ plasmids (Fig. 2). Plasmid pMLB1034 contains the *E. coli* β-galactosidase gene (*lacZ*) (Weinstock et al. 1983). *E. coli* GM48 was used as a host for this plasmid because plasmids are not methylated in this strain (Marinus 1973). This allowed us to restrict pMLB1034 with *BclI* and to ligate the linearized plasmid to the 1.4 kb *Bam*HI fragment carrying the Km^r gene from pKM1. *E. coli* MC1000 transformants were selected on Ap- and Km-containing agar plates. The resulting plasmids pKVZ1 and pKVZ2 contain the Km^r gene in both orientations in the *lacZ* gene.

For the construction of pKVZ11, pKVZ12, pKVZ21 and pKVZ22, the 3.9 kb *Pvu*II fragments from pKVZ1 and pKVZ2 (containing the *lacZ*/Km^r region) were isolated and ligated to *Pvu*II linearized pBR322. Transformants in *E. coli* MC1000 could only be obtained on Ap- and Km- or on Tc- and Km-containing agar plates. No transformants were obtained on agar plates containing Ap, Tc and Km. The resulting plasmids (pKVZ11, 12, 21 and 22) carry the Km^r gene inside the truncated *lacZ* gene in all four possible orientations relative to pBR322. All four plasmids are super multi-copy due to the destruction of copy control by cloning in the *Pvu*II site of pBR322 (see e.g. Twigg and Sherratt 1980).

Results

To test whether *E. coli* mutants could be isolated by a combination of homologous recombination and subsequent plasmid segregation, the *E. coli* glycogen branching enzyme gene (*glgB*) was used as a test system. This gene was chosen because mutants can be easily distinguished; mutant *E. coli* colonies, producing an unbranched (linear) glycogen as a storage polymer, stain blue when flooded with iodine solution, whereas wild-type colonies stain brown (Damotte et al. 1968). We also took advantage of the fact that a large chromosomal insert (about 8 kb) carrying the *E. coli glgB* gene is present on plasmid pOP190. This provides a large region of homology between the plasmid and the *E. coli* chromosome allowing a high rate of homologous recombination. Furthermore, the large size of the insert in the plasmid would probably enhance the segregational instability of the plasmid.

The vector we constructed for this purpose is shown in Fig. 1. Plasmid pKVB2 contains an internally deleted *glg*B gene, which was obtained by replacing a 1.2 kb *Bam*HI fragment in pOP190 by a kanamycin-resistance marker. During the construction of pKVB2 we observed that the plasmid was not stably maintained in *E. coli* JM83. When *E. coli* JM83 (pKVB2) was grown on selective medium (Tc and Km) for more than about 5 generations, plasmid DNA extracted from the cells consisted of a mixture of plasmids. Pure pKVB2 could only be extracted from *E. coli* JM83 by transferring transformed colonies to a small volume of selective medium (2 ml) and allowing growth for less than 5 generations.

We suspected that the structural instability of pKVB2 in JM83 was due to homologous recombination between the *E. coli* chromosome and the plasmid. To test this, pKVB2 was introduced into JM83, and transformants were subcultured for 50 generations in medium supplemented with Tc. Plasmid DNA was extracted from the cells every 10 generations, restricted and analysed by agarose gel electrophoresis. As shown in Fig. 3 the subculturing of *E. coli* JM83 (pKVB2) resulted in replacement of the 1.4 kb *Bam*HI fragment (carrying the Km^r gene) by a 1.2 kb *Bam*HI restriction fragment. Comparison of the restriction fragments in lane M2 and lanes 10–50 (Fig. 3), and further restriction analysis of the plasmid DNA obtained (data not shown) suggested that the plasmid obtained after subculturing contained the complete *glgB* gene and thus was identical to pOP190. This was confirmed by the ability of the plasmid to complement the *glgB* mutation present in *E. coli* KV832 (constructed in this study).

These results strongly suggested that a gene replacement had occurred between the *E. coli* chromosome and plasmid PKVB2 by a reciprocal recombination event, apparantly resulting in the introduction of the mutant gene into the chromosome and the transfer of the intact *glgB* gene to the plasmid. However this recombination did not result in colonies having a mutant phenotype. Apparantly the chromosomal *glgB* mutation was complemented by the intact *glgB* gene, now present on the plasmid. However colonies showing the mutant phenotype were generated after subsequent plasmid segregation as described below.

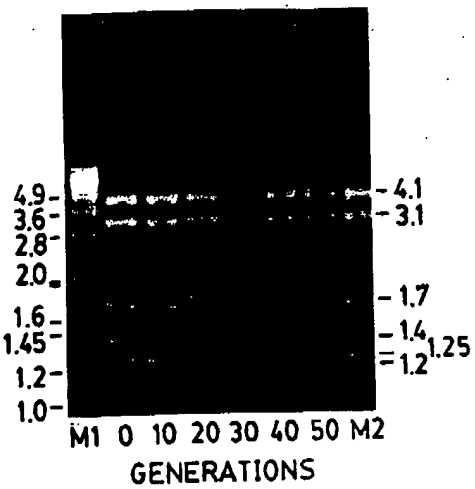

Fig. 3. Analysis of homologous recombination of plasmid pKVB2 in *E. coli* JM83. *E. coli* JM83 harbouring pKVB2 was subcultured for 50 generations in medium containing Tc. Plasmid DNA was extracted from the cells, restricted with *Bam*HI and analysed by agarose gel electrophoresis. Phage SPPI DNA restricted with *Eco*RI (lane M1) and plasmid pOP190 DNA restricted with *Bam*HI (lane M2) were used as markers. Fragment lengths are given in kb

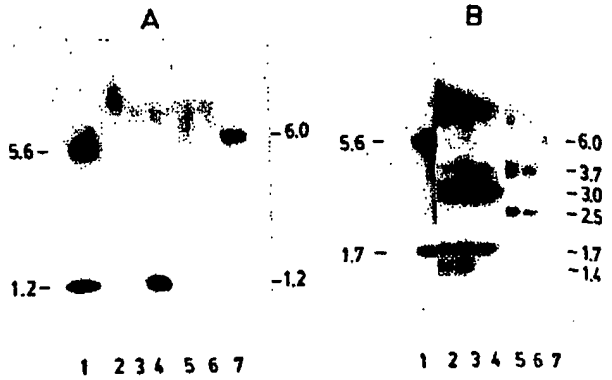

Fig. 5A, B. Southern blot analysis. Lanes 4 and 7, chromosomal DNA from *E. coli* JM83; lanes 2 and 5 and lanes 3 and 6, chromosomal DNA from two Km^r *glgB* mutants constructed via gene replacement with plasmid pKVB2. The DNA was restricted with *Bam*HI (lanes 2–4) or *Hin*dIII (lanes 5–7). Hybridization was performed with A nick-translated pKVB1 and B nick-translated pKVB2. Plasmid pAD1 DNA restricted with *Bam*HI was used as an internal marker (lane 1). As an external marker phage SPPI DNA restricted with *Eco*RI was used (not shown). Known lengths of chromosomal restriction fragments are given

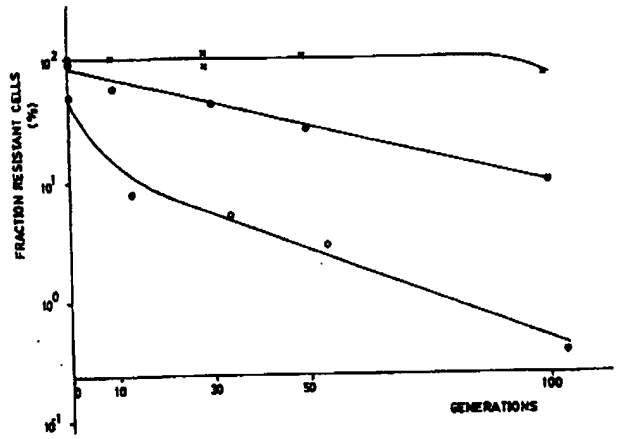

Fig. 4. Segregation kinetics of plasmids pBR322, pOP190 and pKVB2 in *E. coli*. Cells that had been grown for 5 generations in selective medium were diluted into antibiotic-free medium and subcultured for 100 generations. At appropriate times the fractions of plasmid containing cells were determined by plating on TY agar plates containing Tc. (×) pBR322; (●) pOP190; (○) pKVB2

Stability of plasmids pOP190 and pKVB2 in E. coli

During the course of the investigation it was observed that plasmid-free cells were obtained when *E. coli* JM83 (pOP190) was grown without antibiotic selection. This indicated that pOP190 is segregationally unstable in *E. coli*. Quantitative data concerning the segregational loss of the plasmids pOP190, pKVB2 and pBR322 are presented in Fig. 4 and show that, whereas pBR322 was almost stably maintained in *E. coli* during cultivation for about 100 generations without antibiotic pressure, pOP190 and pKVB2 were unstable. pOP190 was rapidly lost from the cells; after 100 generations of growth only about 10% of the cells still contained the plasmid. The segregational kinetics curve obtained for pKVB2 is biphasic; the rate of plasmid loss being very high during the initial stages of culturing, and becoming somewhat lower at later stages.

Construction of a chromosomal mutant with a internal deletion in the glgB gene

We constructed the *E. coli glgB* mutant by replacing the *glgB* region of the chromosome with the *glgB*/Km^r region of pKVB2 via homologous recombination. Due to plasmid loss it is simple to isolate glycogen branching enzyme deficient mutants using the Km^r gene and the iodine staining technique. *E. coli* JM83 was transformed with pKVB2 and was first grown for 10 generations in selective medium, subsequently for 10–50 generations in antibiotic-free medium and then diluted 10^6-fold and plated on medium containing glucose and Km. After staining with iodine solution blue colonies could already be detected after 10 generations of growth without antibiotic pressure. These were toothpicked onto fresh medium and tested for Tc sensitivity. All blue staining, Km^r colonies were Tc^s, probably indicating that the mutants arose by a combination of homologous (reciprocal) recombination and subsequent plasmid loss.

Determination of the genomic structure of E. coli KV832. a glgB deletion mutant

The gene structure of the relevant region of the *E. coli glgB* mutant strains obtained as described above was confirmed by Southern blot analysis of chromosomal DNA isolated from *E. coli* JM83 and two of its Km^r *glgB* mutants. Figure 5 (panel A) shows that when plasmid pKVB1 (see Fig. 1) was used as a probe, hybridization with *Bam*HI or *Hin*dIII digests of wild-type chromosomal DNA gave sig-

nals corresponding to fragments of 1.2 kb and 6.0 kb respectively, as expected (lanes 4 and 7). In contrast no significant hybridization signal was obtained with digests of the mutant chromosomal DNA (lanes 2 and 3 and lanes 5 and 6).

Hybridization was also performed with pKVB2 as a probe (Fig. 5 panel B). This probe hybridized with *Bam*HI and *Hin*dIII digests of the wild-type chromosomal DNA as expected (lanes 4 and 7). However, an extra hybridization signal corresponding to a fragment of 1.4 kb was obtained with *Bam*HI digests of mutant chromosomal DNA (lanes 2 and 3). This result confirms the presence of the Km^r gene on the chromosome. Introduction of this gene into the chromosome via homologous recombination produces an extra *Hin*dIII site in the *glgB* gene region. This explains the two hybridization signals corresponding to fragments of 3.7 kb and 2.5 kb (lanes 5 and 6).

Development of a general system for the simple and efficient isolation of chromosomal E. coli mutants via homologous recombination and plasmid segregation: production of lacZ mutants

The principles used to construct the mutant *E. coli* strain KV832 were extended to develop a general system for the introduction of mutations in any gene of *E. coli*, provided it is dispensable. Ideally such a system should incorporate the easy isolation of mutants even when the mutants can not be distinguished in a simple way, when the rate of plasmid loss is low, and when the region of homology between the plasmid and the chromosome is small. By isolating mutants for the *E. coli* β-galactosidase gene (*lacZ*) we intended to show that these demands could indeed be met.

Figure 2 shows the construction of the vectors needed for this purpose. The plasmids pKVZ11, 12, 21 and 22 all contain a truncated *lacZ* gene due to a deletion of the first 78 bases of the *lacZ* gene by the subcloning of the 3.9 kb *Pvu*II fragment from pKVZ1 or pKVZ2 into pBR322. Since this deletion inactivates the *lacZ* gene, homologous recombination between the *E. coli* chromosome and one of the pKVZ plasmids will directly produce cells with the mutant phenotype because the resulting chromosomal mutation cannot be complemented by the DNA fragment present on the plasmid. A further consequence of the cloning in the *Pvu*II site of pBR322 is the presence of an intact Tc^r gene on the constructs. This allows direct selection of cells that have lost the plasmid; Tc^s cells can be easily isolated on plates containing fusaric acid because they are resistant to this substance. Additionally the construction of the various pKVZ plasmids allowed us to test whether the orientation of the *lacZ* and Km^r genes relative to each other and to pBR322 had an effect on the recombination efficiency and the efficiency of mutant selection.

E. coli C600 (LacZ$^+$) was transformed with pKVZ 11, 12, 21 and 22, transformants were grown for approximately 10 generations in selective medium (Tc and Km) and were subcultured in antibiotic-free medium for 30 generations. The cultures were then plated on TY medium containing Xgal to check whether homologous recombination had occurred, and on fusaric acid selection medium to isolate plasmid-free cells.

Table 2 shows that white colonies were found on Xgal plates indicating that replacement of the chromosomal *lacZ* region by the corresponding *lacZ*/Km^r region on the plasmid had occurred. In the case of pKVZ11 and pKVZ21 the reciprocity of the recombination event could be detected on the plasmid level (data not shown). Furthermore, the number of white colonies observed (Table 2) showed that the orientation of the Km^r gene relative to pBR322 had an effect on the recombination efficiency. In both pKVZ11 and pKVZ21 the orientation of the Km^r gene was clockwise relative to pBR322 and with these plasmids the largest number of white (LacZ$^-$) colonies was found.

Table 2. Effect of the orientations of the Km^r and *lacZ* genes in the pKVZ plasmids relative to each other and to pBR322 on recombination efficiency, segregational instability and efficiency of mutant isolation

Plasmid	Percentage white colonies on Xgal plates[a]	Percentage FA^r colonies[b]	Efficiency of production of *lacZ* mutants[c]
pKVZ11	2%–37%	50%–60%	96%–100%
pKVZ12	approximately 0.1%	2%–45%	93%– 99%
pKVZ21	2%–3%	53%–79%	26%– 75%
pKVZ22	approximately 0.1%	77%–82%	97%– 99%

[a] The percentage white colonies on Xgal plates is a measure of recombination efficiency
[b] The percentage FA^r colonies is a measure of the segregational instability of the plasmid
[c] The percentage of Tc^s colonies amongst the Km^r FA^r colonies, a measure of the efficiency of mutant isolation

The number of fusaric acid resistant colonies found varied considerably (see Table 2) indicating a different degree of segregational instability for the various pKVZ plasmids in *E. coli*. For the isolation of β-galactosidase-deficient mutants amongst the FA^r colonies, we resuspended the colonies in 2 ml TY medium and after dilution replated the cells on medium supplemented with Km. FA^r Km^r colonies should be mutants with the Km^r gene integrated into the *lacZ* gene on the chromosome by gene replacement. Table 2 shows that most of the FA^r Km^r colonies were Tc^s. These colonies were shown to be LacZ$^-$ when tested on medium containing Xgal. All Tc^r colonies apparantly had escaped the fusaric acid selection and still contained the plasmid. Most of these colonies had normal β-galactosidase activity. Table 2 also shows that the orientation of the *lacZ* and Km^r genes relative to each other and to pBR322 had only a small effect on the efficiency of mutant isolation. High efficiencies were obtained even for pKVZ12 and pKVZ22 although these had the lower recombination efficiencies and although in one case only a few FA^r colonies were obtained with pKVZ12.

These results show that *lacZ* mutants can be easily isolated. Confirmation that these mutants resulted from homologous recombination between the chromosome and a plasmid was obtained by Southern blot analysis of chromosomal DNA isolated from two *lacZ* mutants (strains KV6001 and KV6002 obtained via reciprocal recombination with pKVZ11 and pKVZ21 respectively): only the expected restriction fragments hybridized when pKVZ11 was used as a probe (data not shown).

Discussion

The introduction of mutations into chromosomal genes of *E. coli* by gene replacement must meet a number of require-

ments: (i) the gene of interest must be dispensable, (ii) the *E. coli* strain used must be capable of homologous recombination, (iii) the gene of interest must have been cloned. A number of techniques of gene replacements between plasmids, linear DNA fragments or phages and the *E. coli* chromosome have already been described (Gutterson and Koshland 1983; Gay 1984; Jason and Schimmel 1984; Matsuyama and Mizushima 1985; Joyce and Grindley 1984). All techniques make use of circular or linear DNA molecules containing the mutant gene that cannot replicate extrachromosomally in *E. coli*. Mutants are isolated via a selection for cells that have replaced the wild-type chromosomal gene by the mutant gene carrying an antibiotic-resistance marker. A drawback of all these techniques is the need for special *E. coli* strains (e.g. *pol*A; *pol*A^{ts}; *rec*BC*sbc*B), special plasmids (temperature-sensitive origin of replication) or special phages (thermoinducible λ phage lacking the normal *att* site).

This work describes a method of gene replacement that does not need special strains, plasmids or phages for the construction of *E. coli* mutants. A technique that distinguishes mutant cells from wild-type cells (e.g. by colony staining) is helpful, but not required. Our method makes use of the fact that many recombinant, multicopy plasmids are segregationally unstable in *E. coli* (Summers and Sherratt 1984); this allows mutants to be easily isolated by selection for cells in which the mutant gene (marked by an antibiotic-resistance marker) has been integrated into the chromosome and from which the donating plasmid has been lost. We consider plasmid pBR322 ideal for these purposes. The use of this vector has a number of advantages: (i) it is a well-known, commonly used plasmid in which many genes have already been cloned, (ii) it contains a Tc^r gene which allows plasmid-free cells to be easily selected, (iii) recombinant plasmids based on pBR322 are segregationally unstable in *E. coli* (Skogman et al. 1983). We have used our method to construct an internal deletion mutant for the glycogen branching enzyme gene (*glgB*) and mutants for the β-galactosidase gene (*lacZ*).

The results obtained during the construction of the *glgB* deletion mutant showed that mutants isolated resulted from reciprocal recombination between the plasmid and the chromosome. Such a mechanism introduces the mutant gene into the chromosome and transfers the wild-type gene to the plasmid (see Fig. 3). Our system does not allow us to discriminate whether this reciprocal recombination is due to two consecutive crossings-over (integration of the plasmid into the chromosome followed by resolution of the plasmid-chromosome complex) or to two simultaneous cross-over events.

The fact that a chromosomal gene can be transferred to a recombinant plasmid by reciprocal recombination (see Fig. 3) also allows the transfer of chromosomal mutations to multicopy plasmids. Specialized transducing phages have been used for several years to isolate such chromosomal mutations (see Silhavy et al. 1984 for discussion). Only recently have chromosomal mutations been recombined directly onto plasmids carrying the wild-type gene using UV irradiation (Chattoraj et al. 1984), integration in *polA* strains (Saarilahti and Palva 1985) or transduction with phage P1 (Liljestrom et al. 1985).

Figure 6 shows the proposed structure of a plasmid that should be ideal for the isolation of chromosomal mutations. Only a minor change in the plasmid construct we have

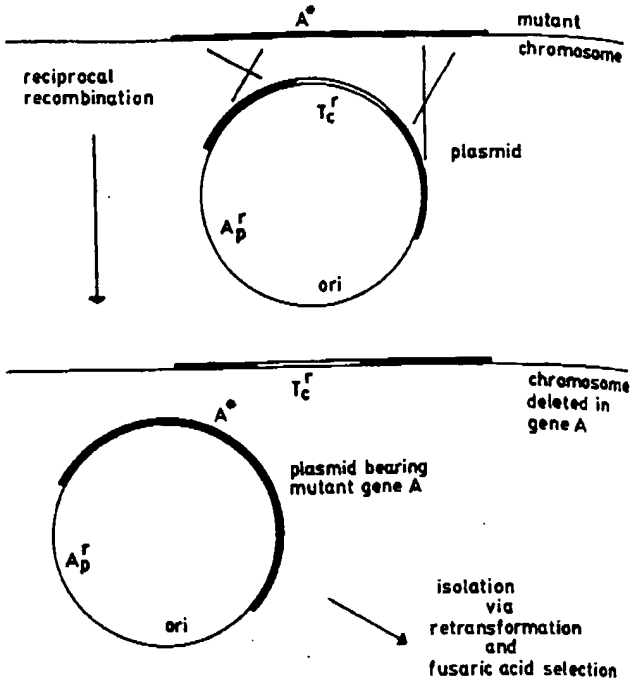

Fig. 6. Moving a chromosomal *E. coli* mutation to a multicopy plasmid via reciprocal recombination. The plasmid is a pBR322 derivative carrying the gene A region of *E. coli* in which the wild-type gene A has been replaced by the Tc^r gene of pBR322. The plasmid is transformed into the mutant *E. coli* strain (the mutation is indicated by an *asterisk*, due to reciprocal recombination, cells that harbour plasmids carrying the mutant gene A will appear. After extraction of total plasmid DNA these plasmids can be easily isolated via retransformation into *E. coli* using Ap and FA selection

used for the isolation of mutants is necessary. Transfer of the Tc^r gene from the plasmid to the mutant chromosome via reciprocal recombination allows a positive selection for plasmids that contain the chromosomal mutation: first plasmid DNA should be extracted from the cells and retransformed into *E. coli*; then Tc^s transformants carrying the plasmid with the mutant gene should be isolated by plating on selective fusaric acid containing plates. As far as we are aware the technique we propose has not been described for *E. coli*, although gene conversion techniques have been described for other micro-organisms (for *Bacillus subtilis* see Iglesias and Trautner 1983).

One possible explanation for the rapid loss of plasmid pKVB2 during the first few generations of growth of JM83 (pKVB2) is that a substantial fraction of cells loses the plasmid during the recombinational exchange of chromosomal and plasmid sequences due to the formation of an abnormal recombination intermediate (homologous sequences, interrupted by non-homologous sequences). In agreement with this hypothesis is the observation that recombination occurred during the phase of rapid plasmid loss.

During the construction of the *lacZ* mutants we also investigated the effect of the orientation of the Km^r gene and the *lacZ* gene relative to each other and to pBR322 on the efficiency of homologous recombination and the efficiency of mutant isolation. The results (Table 2) show that the orientation of the Km^r gene relative to pBR322 had a clear effect on the number of LacZ$^-$ colonies obtained.

Possibly the orientation of the Km^r gene in pKVZ11 and pKVZ21 (clockwise with respect to pBR322) interferes in an unfavourable way with the maintenance of the plasmid, thus favouring the production of cells that have the Km^r gene on the chromosome rather than on the plasmid.

Even though some difference in the efficiency of mutant production with the various pKVZ plasmids was observed (see Table 2), all constructs allowed the isolation of mutants with high efficiency (>30%). Apparantly the orientation of the antibiotic-resistance marker and the gene of interest relative to each other and to pBR322 is relatively unimportant.

The fact that Tc^r colonies were found after fusaric acid selection indicates that this selection technique does not completely guarantee the selection of plasmid-free (Tc^S) colonies as previously noted by Maloy and Nunn (1981). Despite this, the general method described here allows mutants to be simply and efficiently isolated via gene replacement and plasmid loss. Neither special *E. coli* strains nor a technique for the distinguishing of mutants is required.

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